Atherothrombotic risk factor clustering in healthy male relatives of male patients with intermittent claudication

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Objective: **Family history is an independent risk factor for premature acute myocardial infarction; in contrast, familial risk for peripheral arterial disease (PAD) has yet to be determined. Elevated levels of hemostatic proteins are consistently predictive for cardiovascular risk in "healthy" subjects, and may cluster with underlying insulin resistance. Atherothrombotic risk factor clustering occurs in first-degree relatives of subjects with coronary artery disease and type 2 diabetes. These may contribute to the enhanced cardiovascular risk in these subjects, and we hypothesised that familial clustering may occur in PAD. The objective of this study was to measure atherothrombotic risk factors in healthy male first-degree relatives of men with intermittent claudication, with emphasis on thrombotic risk.**

Methods: **One hundred sixty-five healthy male first-degree relatives were compared with control subjects matched for age, sex, and race (n = 165), free from a personal or family history of premature cardiovascular disease. Primary outcome measures were fibrinogen, von Willebrand factor, factor VII clotting activity (FVII:C), and factor XIII levels. Atherosclerotic risk factors were measured, and subjects were genotyped for common functional polymorphisms (factor VII r353q and fibrinogen B β-455).**

Results: **Relatives had higher mean levels of fibrinogen (3.04 vs 2.89 g/L; $P = .021$), FVII:C (117% vs 104%; $P = .000$), factor XIII B subunit (1.11 vs 1.01 IU/mL; $P = .000$), and complex (A_2B_2; 1.18 vs 1.11 IU/mL; $P = .021$). At multivariate analysis the association between relative status and fibrinogen, FVII:C, and factor XIII B subunit levels were independent of other variables.**

Conclusions: **The healthy male relatives of men with PAD have elevated levels of fibrinogen, factor VII, and factor XIII. Our results support the existence of thrombotic risk factor clustering in this population at "high risk." (J Vasc Surg 2004;40:891-8.)**

Atherosclerosis is a generalized disorder that culminates in plaque formation and superimposed thrombosis. Although plaque composition primarily determines vulnerability to disruption,[1] many studies indicate that perturbed hemostasis has a pivotal role in atherothrombosis. In "healthy" subjects elevated levels of fibrinogen,[2,3] factor VII,[4] and von Willebrand factor (vWF)[5] independently predict for the incidence of acute myocardial infraction (AMI). More recently associations between factor XIII and type 2 diabetes,[6] coronary artery disease (CAD), and AMI[7] have been reported. Insulin resistance is an adverse metabolic state characterized by clustering of multiple atherogenic risk factors[8] that constitute insulin-resistance syndrome. Elevated levels of key hemostatic proteins have been described in association with insulin resistance,[9] and support the existence of a "pre-thrombotic" component to this syndrome complex.

Further studies have established family history as an independent risk factor for premature CAD,[10,11] and heritability studies support the causal involvement of genetic factors.[12,13] The familial nature of peripheral arterial disease (PAD) has yet to be investigated in an epidemiologic study. Recent small studies, however, have shown a high prevalence of cardiovascular disease (CVD)[14] and occult PAD[15] in first-degree relatives of subjects with premature PAD. Atherothrombotic risk factor clustering occurs in first-degree relatives of subjects with CAD[16-18] and type 2 diabetes,[6,19] and may contribute to their enhanced cardiovascular risk. We hypothesised that male first-degree relatives of men with intermittent claudication cluster multiple atherothrombotic risk factors. The purpose of this study was to measure these factors, with emphasis on thrombotic risk.

METHODS

This case-control family study was carried out at the United Leeds Teaching Hospitals NHS Trust and the Huddersfield NHS Hospital Trust, West Yorkshire, United Kingdom. All study subjects were recruited from the general population within the geographic location of West Yorkshire.

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Table I. Clinical characteristics of male patients with intermittent claudication (probands)

Patients with intermittent claudication	
Age (y)	62 (57,65)*
Age at diagnosis (y)	54 (50,60)*
Claudication distance (m)	200 (100,250)*
Ankle-brachial pressure index	0.64*
Peripheral vascular interventions	52 of 108 (48%)
Angioplasty or stenting	20/108 (18.5%)
Surgical endarterectomy or bypass†	27/108 (25%)
Both radiologic and surgical intervention	5/108 (4.5%)
Coexistent cardiovascular disease‡	50/108 (46%)
Co-existent diabetes	24/108 (22%)

*Nonparametric data expressed as median (25th, 75th quartiles).
†Bypass (aortoiliac, femorofemoral, femoropopliteal) or common femoral endarterctomy.
‡Coronary artery disease, cerebrovascular disease.

Ethical approval. The study was approved by the Leeds Teaching Hospitals NHS Trust and the Huddersfield NHS Hospital local research ethics committees.

Sample size. A priori sample size calculations were performed for all primary outcome measures, but ultimately were factor VII–dependent. One hundred sixty-five subjects were required per group to demonstrate a 10% difference in factor VII levels for 80% power at the 5% significance level.

Subjects. One hundred eight subjects with intermittent claudication (probands) were identified from consecutive patients attending the vascular outpatient department. All were white men younger than 65 years. All probands had intermittent claudication as defined by the Edinburgh Claudication Questionnaire,[20] with stable symptoms for more than 6 months, and ankle-brachial pressure index (ABPI) less than 0.8. Review of the clinical notes showed that 60 patients (56%) had atherosclerotic PAD confirmed at angiography, and 48 patients (44%) had atherosclerotic pad confirmed at duplex scanning. Subjects with critical limb ischemia were excluded to maintain the homogeneity of the study population. Overall, 50 patients (45%) had concomitant CVD, and 52 patients (48%) had undergone previous peripheral vascular intervention (Table I). Of these subjects, 20 (18.5%) underwent angioplasty or stenting, 27 (25.0%) underwent open surgical intervention, and 5 (4.6%) underwent both radiologic and surgical intervention.

From these probands 165 male first-degree relatives were recruited, of whom 37 (22.4%) were brothers and 128 (77.6%) were sons. In light of known sex differences in hemostatic parameters, only male subjects were recruited, to maximize recruitment and maintain the power of the study. All first-degree relatives were between 18 and 65 years of age, and were free from clinical CVD and known diabetes. These were compared with control subjects matched for age, sex, and race recruited at random from the Leeds Family Health Authorities Register. All control subjects were free from clinical CVD, diabetes, and a family history of CVD (defined as <65 years for the purpose of the study).

Clinical assessment. All subjects provided informed consent, per protocol approved by the relevant local research ethics committee. All subjects fasted for 10 hours overnight, and were asked to refrain from smoking during this time. All subjects were free from both intermittent claudication and angina, according to Edinburgh Claudication[20] and Rose Angina Questionnaires.[21] A detailed medical history was taken, including drug history and consumption of nicotine and alcohol. Blood pressure was determined with an automated cuff to the nearest 2 mm Hg, and taken as the mean of 3 readings. Body mass index (BMI) was calculated as weight in kilograms divided by height-squared in meters (kg/m^2). Waist-hip ratio (WHR) was calculated as minimal abdominal girth (to the nearest 0.5 cm) divided by maximal protrusion of the hips (in centimeters) at the level of the symphysis pubis. Twelve-lead electrocardiography was performed to exclude myocardial ischemia or previous infraction. Resting ABPI was measured in both legs with a hand-held 8-MHz Doppler probe (Handydop; SciMed) over the infragenicular arteries. ABPI of less than 0.9 is 95% sensitive in detecting angiogram-positive disease, and ABPI 0.9 or greater is almost 100% specific in detecting "healthy" subjects.[22] All subjects with ABPI less than 0.9 were excluded from the study. Fifty milliliters of free-flowing venous blood was drawn from an antecubital vein with a 19-gauge needle. Blood was transferred to a room temperature lithium heparin tube for plasma lipid profile, lithium fluoride for plasma glucose assay, ethylenediamine tetraacetic acid for deoxyribonucleic acid extraction, and sodium citrate for hemostatic proteins. A pre-cooled lithium heparin tube was used for plasma insulin assay, and was transferred immediately onto ice. All subjects underwent a 75-g oral glucose tolerance test, and plasma glucose concentration was determined at 2 hours. Glycemic state was classified in accordance with World Health Organization criteria.[23]

Laboratory methods. Sample tubes at room temperature were centrifuged at 3000*g* (MSE; Sanyo Gallenkamp) for 20 minutes. Iced samples were centrifuged at 4000*g* for 30 minutes in a pre-cooled centrifuge (Coolspin; Fisons Scientific) at 4° C. Plasma aliquots were snap-frozen in liquid nitrogen before being placed in long-term storage at −40° C. All assays were performed in duplicate, and mean levels were calculated. Plasma fibrinogen was measured with the Clauss method.[1] Factor VII clotting activity (FVII:C) was performed on an ACL 300 Plus (Instrumentation Laboratory) with factor VII–deficient plasma (Sigma) and recombinant rabbit thromboplastin (Instrumentation Laboratories). Levels are expressed as a percentage of activity given by calibration plasma (FVII:C [%]). vWF antigen levels were determined with an enzyme-linked immunosorbent assay with commercially available antibod-

ies (DAKO). Factor XIII B subunit and complex (A_2B_2) antigen levels were determined with an enzyme-linked immunosorbent assay as described.[2] The interassay and intra-assay coefficients of variation for fibrinogen, FVII:C, vWF, factor XIII B subunit, and factor XIII A_2B_2 were 3.5% and 2%, 3.2% and 3.2%, 4.7% and 2.8%, 9.8% and 6.2%, and 9.3% and 5.4%, respectively. Human insulin was measured with an immunoassay (Medgenix-Ins-Easia; (Biosource Europe), and the interassay and intra-assay coefficients of variance were 3.5% and 2.5%, respectively. Insulin resistance was estimated with Homeostasis Model Assessment (HOMA), In which insulin resistance = Fasting glucose (mmol/L) × Fasting insulin (IU/mL)/22.5. This assumes that healthy subjects with normal weight and younger than 35 years have 100% B-cell function and insulin resistance of unity.[26]

Genomic DNA was extracted from leukocytes with a nucleon DNA extraction kit (Nucleon Biosciences). Genotyping was performed for common polymorphisms known to influence hemostatic protein levels, including the fibrinogen β-455 G/A (classified G/G, G/A, or A/A)[3] and factor VII R353q polymorphisms (classified G/G, G/A, and A/A).[4] Plasma glucose was measured with the glucose oxidase method, total cholesterol and triglyceride levels with a Hitachi 747 automatic analyzer (Boehringer-Mannheim), high-density lipoprotein cholesterol with a Hitachi 717 automatic analyzer (Boehringer-Mannheim), and low-density lipoprotein cholesterol with the Friedewald equation.

Statistical methods. Parametric data are presented as mean (standard deviation), log-normal data as geometric mean (anti-logged 95% confidence intervals), nonparametric data as median (25th, 75th percentiles), and categorical data as a proportion (%). An independent Student *t* test was used to assess for a difference in means, Mann-Whitney *U* test for a difference in ranks, and χ^2 test for difference in proportions between study groups. One-way analysis of variance was used to compare hemostatic protein levels according to genotype. Bivariate correlation was used to establish association between hemostatic proteins and other continuous variables. Pearson correlation coefficients (τ) were calculated if both variables were normally distributed, and the Spearman rank coefficient (ρ) was calculated if one or more variables were nonparametric. Forward stepwise multiple linear regression was used to assess for association between hemostatic protein level and relative status, adjusting for significant correlates. Only covariates that independently predicted hemostatic protein levels were included in the final model. The exclusion of nonsignificant correlates did not affect the variance explained by the model (R^2). Adjusted levels of hemostatic protein levels were calculated, allowing for confounding variables. All analyses were performed by one of the authors (D.J.P.) with SPSS for Windows, version 9.0, and $P < .05$ was considered statistically significant.

RESULTS

Clinical and biochemical characteristics

The clinical characteristics of the probands are shown in Table I. Both the first-degree relatives and control subjects were similar in age, alcohol intake, and anthropometric characteristics (Table II). Conversely, the relatives clustered multiple atherogenic risk factors, including higher systolic blood pressure, plasma cholesterol, low-density lipoprotein cholesterol, triglycerides, fasting insulin, and estimated insulin resistance, and higher prevalence of both smoking and impaired glucose regulation.

Fibrinogen. Fibrinogen levels were significantly higher in the first-degree relatives than in control subjects (Table II), and in smokers than in nonsmokers (geometric mean, 3.12 g/L vs 2.87 g/L; $P = .001$). Bivariate analysis showed significant correlation between Log_{10} fibrinogen, age, and vWF levels. In the control group alone there were additional correlations with BMI and diastolic blood pressure (Table III). There was no difference in genotype frequency at the B-455 polymorphism between relatives (GG 107, GA 49, AA 7) and control subjects (GG 112, GA 44, AA 4) ($\chi^2 = 1.173$; 2 degrees of freedom (df); $P = .556$). Nor was there an association between B-455 genotype and fibrinogen levels (relatives, GG 3.07, GA 2.95, AA 3.36 g/L, $P = .339$; controls, GG 2.87, GA 2.85, AA 2.76 g/L, $P = 0.920$). Multiple linear regression was performed with Log_{10} fibrinogen as the dependant variable, and age, smoking status, case type (relative vs control subject), and vWF level as covariates. The final model accounted for 16.4% of variance in Log_{10} fibrinogen levels, with relative status, current smoking status, age, vWF levels, and FVII:C activity independently predicting Log_{10} fibrinogen levels (Table IV). Adjusted fibrinogen levels were significantly higher in first-degree relatives (geometric mean, 3.09 g/L; anti-logged 95% confidence interval [CI], 2.99-3.18) than in control subjects (2.95 g/L; 95% CI, 2.86-3.04; $P = .049$).

Factor VII:C. FVII:C was significantly higher in first-degree relatives than in control subjects (Table II). There was no significant difference in FVII:C between smokers and nonsmokers (mean, 111.4% vs 109.7%; $P = .570$). FVII:C correlated with age and a number of features of insulin-resistance syndrome, including BMI, waist-hip ratio, Log_{10} systolic blood pressure, total cholesterol, and Log_{10} triglyceride levels (Table III). In the first-degree relatives alone there were additional correlations with diastolic blood pressure, Log_{10} heart rate, fasting glucose concentration, and Log_{10} HOMA. There was no difference in genotype frequency at the factor VII R353q polymorphism between first-degree relatives (GG 131, GA 29, AA 3) and control subjects (GG 122, GA 34, AA 2; $\chi^2 = 0.839$; 2 df; $P = .657$). There was, however, a significant association between R353q genotype and FVII:C, with a reduction in activity as the number of A alleles increased (first-degree relatives, GG 119.2%, GA 106.7%, AA 96.0%, $P = .046$; control subjects, GG 108.0%, GA 91.2%, AA 53.0%, $P = .000$).

Table II. Clinical, biochemical, and hemostatic characteristics of study groups

	Relatives (N = 165)	*Control subjects (N = 165)*	P
Age (y)	38 (34,43)	40 (33,48)	.304*
BMI (kg/m^2)	26.3 (4.1)	26.2 (3.8)	.728†
Waist-hip ratio	0.90 (0.07)	0.90 (0.06)	.455†
Systolic blood pressure (mm Hg)	135 (132,137)	130 (128,132)	.006‡
Diastolic blood pressure (mm Hg)	82 (11.5)	80 (11)	.130†
ABPI	1.13 (0.08)	1.13 (0.10)	.766†
Heart rate (bpm)	67.4 (65.5,69.3)	66.4 (64.7,68.1)	.423‡
Alcohol (units/wk).	20 (6,28)	15 (6,30)	.398*
Current smoker (%)	73 of 165 (44%)	51 of 165 (31%)	.012§
Cholesterol (mmol/L)	5.77 (1.14)	5.22 (0.97)	.000†
LDL (mmol/L)	3.68 (1.02)	3.23 (0.89)	.000†
HDL (mmol/L)	1.23 (1.17,1.27)	1.29 (1.23,1.33)	.050‡
Triglyceride (mmol/L)	1.62 (1.47,1.78)	1.34 (1.22,1.47)	.005‡
0-Hour glucose level (mmol/L)	4.90 (4.70,5.30)	5.00 (4.60,5.35)	.662*
2-Hour glucose level (mmol/L)	5.28 (5.01,5.58)	5.23 (5.02,5.44)	.729‡
IGR or diabetes (%)	22/165 (13.3%)	8/165 (4.8%)	.012§
Fasting insulin (μm/L)	8.43 (7.82-9.08)	6.93 (6.58-7.30)	.000‡
HOMA	1.85 (1.70-2.02)	1.53 (1.44-1.62)	.000‡
Fibrinogen (g/L)	3.04 (2.93-3.15)	2.88 (2.79-2.96)	.021‡
FVII:C (%)	116.7 (28.5)	104.1 (21.4)	.000†
vWF (iu/ml)	1.10 (0.45)	1.06 (0.41)	.475†
Factor XIII A_2B_2 (μg/mL)	1.18 (0.28)	1.11 (0.29)	.021†
Factor XIII B subunit (μg/mL)	1.11 (0.23)	1.01 (0.22)	.000†

BMI, Body mass index; *ABPI,* ankle-brachial pressure index; *LDL,* low-density lipoprotein; *HDL,* high-density lipoprotein; *IGR,* impaired glucose regulation; *HOMA,* estimated insulin resistance; *FVII:C,* factor VII clotting activity; *vWF,* von Willebrand factor.

*Nonparametric data expressed as median (25th, 75th quartiles).

†Parametric data given as mean (SD).

‡Log_{10} normal data expressed as geometric mean (anti-logged 95% confidence intervals).

§Categorical data expressed as a proportion (%).

Table III. Bivariate correlation coefficients for fibrinogen, FVII:C, and vWF

	Log $_{10}$ fibrinogen		*FVII:C (%)*		*Factor XIII A_2B_2*		*Factor XIII B subunit*	
	Relatives	*Control subjects*	*Relatives*	*Control subjects*	*Relatives*	*Control subjects*	*Relatives*	*Control subjects*
Age*	0.284	0.255	0.223	0.260	0.175	0.271	0.126	0.406
Body mass index	0.059	0.165	0.217	0.246	-0.043	0.214	0.194	0.301
Waist-hip ratio	0.030	0.151	0.158	0.183	-0.063	0.327	0.057	0.458
Log_{10} systolic blood pressure	0.017	0.079	0.187	0.166	0.029	0.240	0.069	0.201
Diastolic blood pressure	0.009	0.122	0.185	0.140	0.051	0.169	0.069	0.237
Log_{10} heart rate	-0.046	0.142	0.241	0.120	0.121	0.053	0.132	0.121
Alcohol intake*	-0.016	-0.010	-0.015	-0.024	0.164	0.164	0.143	0.037
Cholesterol	0.146	0.119	0.286	0.238	0.157	0.360	0.310	0.292
Log_{10} HDL	-0.078	-0.141	-0.003	0.209	0.020	0.020	0.020	-0.070
Log_{10} triglycerides	0.017	0.141	0.329	0.182	0.210	0.315	0.256	0.394
Fasting glucose*	0.153	0.138	0.277	0.029	0.036	0.335	0.088	0.207
Log_{10} HOMA	0.128	0.134	0.217	0.114	0.156	0.189	0.039	0.294
Log_{10} fibrinogen	1.000	1.000	0.130	0.141	0.125	0.094	0.252	0.220
FVII:C (%)	0.130	0.178	1.000	1.000	0.216	0.206	0.302	0.308
Factor XIII A_2B_2	0.125	0.094	0.216	0.206	1.000	1.000	0.389	0.635
Factor XIII B subunit	0.252	0.220	0.302	0.308	0.389	0.635	1.000	1.000

Note that where both variables are normally distributed, values presented are Pearson correlation coefficients (τ). Where one variable is nonparametric (*), Spearman rank correlation coefficients (ρ) are given. For all data, $P < .05$ where $r \geq 0.160$, $P < 0.01$ where $r \geq 0.200$, $P < 0.001$ where $r \geq 0.256$.
FVII:C, Factor VII clotting activity; *vWF,* von Willebrand factor; *HDL,* high-density lipoprotein; *HOMA,* estimated insulin resistance.

Multiple linear regression was performed with FVII:C as the dependant variable, and case type, nonpossession of the r353q A allele (ie, GG vs GA or AA), and other correlates as covariates. The final model accounted for 25.8% of variance in FVII:C levels (Table V). Adjusted levels of FVII:C were significantly higher in first-degree relatives (mean, 109.4%; 95% CI, 105.4-113.5) than in control subjects (100.8%; 96.8-104.9; $P = .001$).

Table IV. Multiple linear regression model for Log_{10} fibrinogen: Parameter, effect size, and contribution to variance

Parameter	*Regression coefficient (95% CI)*	*Effect**	*Contribution to variance (%)*	P
Final model			16.4	.000
Relative status	$0.02\ (6.65^{-05}\text{-}3.81^{-02})$	0.20	1.1	.049
Current smoker	$0.03\ (1.01^{-02}\text{-}4.91^{-02})$	0.30	2.5	.030
Age (y)	$1.40^{-03}\ (3.14^{-04}\text{-}2.48^{-03})$	0.14	2.0	.120
vWF antigen	$6.35^{-02}\ (2.69^{-02}\text{-}1.00^{-01})$	0.07	2.5	.002
FVII:C	$6.30^{-04}\ (2.44^{-02}\text{-}1.01^{-01})$	0.06	2.8	.001

$R^2 = 0.164$ (adjusted $R^2 = 14.8$).
vWF, von Willebrand factor; *FVII:C,* factor VII clotting activity.
*Change in fibrinogen (g/L) according to relative vs control status, current smoker vs nonsmoker status, 10-year increase in age, 0.1 IU/mL increase in vWF, and 10% increase in FVII:C.

Table V. Multiple linear regression model for FVII:C: Parameter, effect size, and contribution to variance

Parameter	*Regression coefficient (95% CI)*	*Effect**	*Contribution to variance (%)*	P
Final model			25.8	.000
Nonpossession A allele (M2)	17.55 (11.40-23.71)	17.3	7.8	.000
Relative status	8.61 (3.36-13.86)	8.6	2.5	.001
Cholesterol	3.97 (1.18-6.77)	4.0	1.9	.005
Body mass index	0.81 (0.13-1.50)	4.1	1.3	.020
Age (y)	0.30 (0.09-0.60)	3.6	1.0	.043
Log triglycerides	11.60 (0.05-23.1)	1.2	1.0	.049

$R^2 = 0.258$ (adjusted $R^2 = 0.241$).
FVII:C, Factor VII clotting activity.
*Change in FVII:C activity (%) according to relative vs control status, nonposssession of A allele, 1-mmol/L increase in cholesterol, 5-kg/m^2 increase in body mass index, and 10-year increase in age.

Factor XIII A_2B_2. FXIII A_2B_2 levels were significantly higher in the first-degree relatives compared with control subjects (Table I) and in smokers compared with nonsmokers (mean, 1.24 vs 1.09 μm/mL; $P = .000$). FXIII A_2B_2 significantly correlated with a number of features of insulin-resistance syndrome, including age, alcohol intake, total cholesterol, Log_{10} triglyceride level, FVII:C, Log_{10} HOMA, and the B subunit. Additional correlations with BMI, waist-hip ratio, and Log_{10} systolic and diastolic blood pressure were present in control subjects. The final multiple linear regression model accounted for 31.5% of variance (R^2) in factor XIII A_2B_2 levels. Only the factor XIII B subunit, current smoking, Log_{10} plasminogen-activator inhibitor-1 triglyceride, and Log_{10} HOMA independently predicted factor XIII A_2B_2 levels. There was no difference in adjusted factor XIII A_2B_2 levels between first-degree relatives (mean 1.16 μm/mL; 95% CI, 1.12-1.20), and control subjects (1.15 ng/mL; 95% CI, 1.11-1.19; $P = .694$).

Factor XIII B subunit. Factor XIII B subunit levels were significantly higher in first-degree relatives than in control subjects (Table II) and in smokers than in nonsmokers (mean, 1.10 vs 1.03 μm/mL; $P = .05$). B subunit levels correlated with a number of features of insulin-resistance syndrome (Table III), including BMI, total cholesterol, Log_{10} triglycerides, Log_{10} fibrinogen, FVII:C, Log_{10} PAI-1, and factor XIII A_2B_2 levels in both study groups. The final regression model accounted for 36.3% of variance in B subunit levels (Table VI). Adjusted factor XIII B subunit levels were significantly higher in first-degree relatives (mean, 1.08 ng/mL; 95% CI, 1.06-1.11) than in control subjects (1.04 ng/mL; 95% CI, 1.01-1.07; $P = .024$).

vWF. There was no significant difference in vWF levels between first-degree relatives and control subjects. Only age and Log_{10} fibrinogen independently predicted vWF levels ($\beta = 0.010$, $P = .001$, and $\beta = 0.719$, $P = .037$; $R^2 = 8.9\%$, respectively) in the final regression model.

DISCUSSION

This is the first study to show that "healthy" male relatives of men with intermittent claudication cluster multiple clinical, biochemical, and metabolic risk factors for atherosclerosis. The emphasis of this study was on thrombotic risk, however, and our novel findings demonstrate elevated plasma fibrinogen, factor VII, and factor XIII levels in these subjects. Although case-control studies are prone to bias and confounding, the robust method used for our study reinforces the validity of our findings. First, the presence of lower limb atherosclerosis in all probands was determined both clinically and radiologically, thus providing an unequivocal family history of PAD. Second, to ameliorate recruitment bias, we synchronously recruited well-characterized and directly comparable first-degree relatives and control subjects from the general population of West Yorkshire. Ultimately 78% of all probands with an

Table VI. Multiple linear regression model for factor XIII B subunit: Parameter, effect size, and contribution to variance

Parameter	*Regression coefficient (β) (95% CI)*	*Effect size*	*Contribution to variance (%)*	P
Final model			36.3	.000
Factor XIII A_2B_2	0.337 (0.262-0.411)	0.34	15.8	.000
FVII:C	1.24^{-02} (3.83^{-04}-2.10^{-03})	0.12	1.6	.005
Body mass index	7.46^{-03} (1.98^{-03}-1.29^{-02})	0.04	1.4	.008
Log_{10} triglyceride	0.109 (0.241-0.195)	0.11	1.3	.012
Relative status	4.78^{-02} (6.38^{-03}-8.93^{-02})	0.05	1.0	.024

R^2 = 0.363 (adjusted R^2 = 0.355).
FVII:C, Factor VII clotting activity.
*Change in factor XIII B subunit level (μg/mL) according to 1-μg/mL increase in factor XIII A_2B_2 levels, 10% increase in FVII:C activity, 5.0-kg/m^2 increase in body mass index, 1-mmol/L increase in triglyceride, and relative vs control status.

appropriate male first-degree relative participated in our study, and all study subjects had clinical CVD excluded with validated and reproducible means. All laboratory assays were performed in duplicate, and our strict quality control measures ensured excellent reproducibility. In addition, to mitigate against confounding we adjusted for a comprehensive array of clinical, biochemical, and metabolic risk factors. Despite these adjustments, the association between relative status and fibrinogen, factor VII, and factor XIII B subunit levels remained significant. The independent nature of this association may be explained by the inheritance of independent genetic factors. Certainly heritability studies suggest that between 33% and 44%, 53% and 62%, and 41% of variance in fibrinogen, factor VII, and factor XIII B subunit levels, respectively, are due to genetic factors.[13,29,30] In our study genetic variation at the fibrinogen B-455 or factor VII r353q polymorphisms did not explain the observed differences in protein levels. Nevertheless, CVD is a polygenic disorder, and this study was not powered as a primary gene association study. Undetermined genetic factors may therefore modulate hemostatic protein phenotype and hence vascular risk within our families. One limitation of our case-control design, however, is inability to differentiate between the effects of genetics and shared environment. "Unrecognized" environmental factors may therefore contribute to protein phenotype in our study population. Furthermore, we cannot fully exclude the presence of subclinical atherosclerotic burden with questionnaire, electrocardiography, and ABPI alone, and these factors may confound our observations. Ultimately our final models explained 16.4%, 25.8%, and 36.3% of variance in fibrinogen, FVII:C, and fctor XIII B subunit levels, respectively, which is consistent with other studies.[6,17-19] Consequently a significant proportion of intersubject variation remains unexplained, and may be due to unknown environmental factors, underlying inflammation, measurement error, or true random variation. Finally, we acknowledge that family history was not the strongest predictor of hemostatic protein levels in our study population. Features of insulin-resistance syndrome were important determinants of FVII:C and factor XIII B subunit levels, whereas fibrinogen was primarily influenced by age, smoking status, and other hemostatic parameters. These data illustrate the complex interplay of genetic and environmental factors that govern protein phenotype, although we stress the importance of heritability studies when quantifying genetic contribution.

Fibrinogen. Fibrinogen is a 340-kd glycoprotein synthesised by hepatic parenchymal cells. Fibrinogen is demonstrable within the tunica intima of atherosclerotic plaque, and the constant cycle of fibrin turnover has been implicated in lesion progression.[31] In healthy subjects elevated fibrinogen independently predicts development of PAD,[4] AMI,[5,6] and stroke.[7] Fibrinogen is thus a powerful pro-thrombotic marker, and this effect is independent of blood viscosity.[34] The mechanisms that underpin these associations remain unclear, although abnormal fibrin gel architecture is associated with AMI in young men.[35] High plasma fibrinogen levels are associated with enhanced factor XIII activation rate and alteration in fibrin clot structure and function.[36] Consequently the high levels in our first-degree relatives may have adverse cardiovascular implications. It is thus interesting that our findings parallel those in other population subgroups at high risk, including the healthy first-degree relatives of patients with severe CAD[18] and type 2 diabetes.[19] Fibrinogen is an "acute phase reactant," however, and the high levels seen during infection and inflammation may be mediated by interleukin-6.[37] Mounting evidence links inflammation to atherothrombosis.[38] The high fibrinogen levels in our relatives may therefore serve as a marker for underlying inflammation or subclinical atherosclerosis. Although the ABPI is a valid and reproducible means of detecting subclinical PAD,[22] it is only accurate in detecting flow-limiting stenoses and not early plaque formation or minimal stenoses. The measurement of subclinical atherosclerotic burden with carotid, aortic, and femoral ultrasound scanning was, however, not the primary purpose of this study, but will form the basis of ongoing investigations.

Factor VII. Factor VII is synthesized by the liver, circulates in the plasma at low levels, and is activated by contact with tissue factor.[39] Elevated FVII:C levels independently predict for AMI in some[4] but not all studies,[2] and may more specifically predict for fatal events.[3] In the

Edinburgh Artery Study elevated FVII:C levels were not associated with development of intermittent claudication,[32] but with symptoms developing in only 52 subjects this study was most certainly underpowered. Elevated FVII:C levels, however, predict for acute cardiovascular events in patients with established PAD,[40] and the presence of elevated levels in our first-degree relatives may likewise confer adverse risk. Although a causal atherothrombotic role has yet to be established, similar findings are demonstrable in the healthy first-degree relatives of patients with type 2 diabetes[19] but not severe CAD.[18] The precise reasons for these discrepancies remain obscure, although PAD should be considered a marker for systemic atherosclerotic burden. In our study about 45% of probands had concomitant CAD, whereas only 10% of Mill's coronary probands had symptomatic PAD. Consequently it may be appreciated that our first-degree relatives were derived from a proband population with advanced systemic atherosclerosis. It is thus plausible that familial mechanisms, including those regulating FVII:C levels, may therefore be more likely to be operative in patients with PAD. Finally, our data support association between factor VII, insulin resistance,[41] and triglyceride level,[42] as previously described. The association between factor VII and relative status was independent of these variables, however, which suggests influence from independent genetic or environmental factors.

Factor XIII. Factor XIII circulates in plasma as an inactive tetramer (factor XIII A_2B_2). The A subunit contains the active motif, and is synthesised in the bone marrow, whereas the hepatic-synthesized B subunit acts as a carrier protein, with up to 50% circulating in the free dimeric form.[43] After thrombin activation, factor XIII catalyses the formation of stable fibrin clot with enhanced mechanical strength and resistance to fibrinolysis.[44] Increased plasma levels of fibrin polymers[45] and abnormal fibrin clot structure and function are present after AMI.[35] Increased plasma fibrin stabilizing activity is demonstrable in atherosclerotic PAD, and correlates with factor XIII levels.[46] More recently, elevated A and B subunit levels have been shown in type 2 diabetes,[6] severe CAD, and AMI.[7] Furthermore, factor XIII inhibition augments the effect of tissue plasminogen activator (t-PA)–thrombolysis in animal models.[44,47] These data suggest that factor XIII has a pivotal role in stable fibrin clot formation, and may have a putative role in the pathogenesis of atherothrombosis. In keeping with our data, elevated factor XIII subunit antigen levels are present in the first-degree relatives of patients with type 2 diabetes.[6] Our data support an association between factor XIII and underlying insulin resistance as consistent with recent observations.[6,17] Thrombotic risk factors cluster in the presence of insulin resistance[9], and factor XIII may represent another dimension to this syndrome complex. The association between relative status and factor XIII A_2B_2 levels was attenuated by adjustment for the B subunit, insulin resistance, triglycerides, and smoking. Thus differences in factor XIII A_2B_2 levels may be partly mediated by insulin resistance, although our cross-sectional study design does not enable us to establish a causal relationship. Conversely, association with the B subunit was independent from other variables. Similarly only B subunit levels are elevated in healthy first-degree relatives of probands with severe CAD.[17] Inasmuch as the B subunit does not contain the active enzymatic site, the functional consequences of our observations are uncertain. Like fibrinogen, the B subunit is synthesized by the liver, however, and the elevated B subunit levels found may simply serve as a marker for chronic inflammation.

SUMMARY

This is the first study to demonstrate the clustering of multiple atherothrombotic risk factors in healthy male relatives of men with intermittent claudication. The emphasis of this study was on thrombotic risk, however, and we have shown increased levels of fibrinogen, factor VII, and factor XIII in our population of first-degree relatives. These findings are directly comparable with other population subgroups, including first-degree relatives of subjects with CAD[16-18] and type 2 diabetes,[6,19] who are at high cardiovascular risk. Elevated hemostatic protein levels consistently predict for cardiovascular risk in healthy subjects, and our findings have identified a pre-thrombotic state in these subjects. Recent data suggest a high prevalence of clinical CVD and occult PAD in first-degree relatives of subjects with premature PAD.[14-15] Our data support the hypothesis that this subgroup of the population is at high risk for CVD. Further longitudinal studies are required, however, to examine the relationship between the pre-thrombotic state and CVD. Once established, this may have major implications for both primary prevention and development of new therapeutic strategies.

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